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Characterization of collagen matrices crosslinked using microbial transglutaminase

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Abstract

In search of a new approach for crosslinking collagen-based biomaterials, we examined the effect of microbial transglutaminase (MTGases) as a crosslinking reagent on collagenous matrices made from porcine type I collagen. As the results revealed, MTGase exhibited a crosslinking action that raised the viscosity of the collagen solution. Matrices crosslinked with MTGase at the low pH values of pH 3 and 4 exhibited higher tensile strengths than those at high pH values. In comparison with untreated matrices, the denaturation temperatures of the corresponding matrices shifted toward higher temperatures. These enzyme-catalyzed crosslinked matrices were proven by MTT assay to be non-cytotoxic. In conclusion, this enzymatic method of using MTGase provides an alternative potential way for crosslinking collagen-based matrices.

Keywords: Transglutaminase; Collagen; Matrix; Enzyme; Crosslinking; Biomaterials

1. Introduction

Collagen, the main structural proteins accounting for the structural integrity of vertebrates and many other multicellular organisms, has been extensively applied to the field of tissue engineering [1–5]. Although collagen is recognized as a promising material, concerns remain about the vulnerability to in vivo enzyme degradation and the low mechanical strength of untreated collagen. In general, collagen-based biomaterials require some chemical treatments such as glutaraldehyde, diphenylphosphorylazide (DPPA), carbodiimides, etc., in order to meet the demands of long-term clinical use [6–11]. Considering the potential cytotoxicity and calcification in some applications among these chemical reagents, on the other hand, there are many physical methods for crosslinking which have been employed such as dehydrothermal (DHT) treatment, photo-oxidation, and microwave and ultraviolet irradiation [12].

Transglutaminases (TGases; protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) are widely distributed in various organisms, including vertebrates, invertebrates, plants, and microorganism, and are reportedly responsible for certain biological events such as epidermal keratinization, blood coagulation, and regulation of erythrocyte membranes. TGases catalyze an acyl-transfer reaction in which γ-carboxamide groups of peptide-bound glutamine residues act as the acyl donor and, generally, the ε-amino groups of lysine residues or some naturally occurring primary amino groups are the acyl acceptor. Thus the polymerization of proteins can be achieved as a result of the formation of intermolecular or intramolecular ε-(γ-glutamyl)lysine bonds [13]. Recently, a microbial transglutaminase (MTGase) isolated from the culture medium of *Streptoverticillium mobaraense* has become commercially available. Unlike TGases from many other sources, the MTGases possess many features, including Ca^{2+} independence, a broader substrate specificity for acyl donors, a smaller molecule size, and a higher reaction rate, which make them suitable for industrial applications [14]. Currently, this

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MTGase has been successfully applied in the food industry for improving the physical properties and texture of protein-related foods like yogurt, tofu, and suwari [15]. More recently, the use of MTGase in many other fields has also been reported [16,17]. Seitz et al. reported the selective crosslinking of bacteriorhodopsin in purple membrane form by MTGase under mild conditions [18]. Catalyzed by MTGase, new gelatin-based hydrogels were crosslinked and obtained from a mixture of purified gelatin and hyaluronan derivatives [19]. Chen et al. compared the ability of the two enzymes, MTGase and tyrosinase, to catalyze the formation of new gels from a mixture of gelatin and chitosan [20].

Therefore, in this study, we examined the effect of MTGase as a crosslinking reagent on collagenous matrices made from porcine type I collagen. For this purpose, the influences of collagen solutions of various pH values, incubation temperatures, and enzyme concentrations on the resultant matrices were investigated. Further, for safety of clinical use, the cytotoxicities of the resultant matrices were also examined.

2. Materials and methods

2.1. Preparation of enzymatic crosslinked collagen matrices

MTGase derived from *Streptoverticillium* was a kind gift from Ajinomoto (Japan). As determined by a colorimetric hydroxamate method [21], the enzyme activity of MTGase was 592 U/g of powder. Type I collagen was prepared from porcine skin by a method described in our previous paper [22]. In order to estimate the effect of the enzyme concentration, pH value of the collagen solution, and incubation temperature during enzyme processing, treatments were divided into groups with different conditions. Collagens were dissolved in 0.01 M acetic acid solutions of various pH values (pH 3, 4, 4.5, 5, and 6) to prepare a final concentration of 10 mg/mL, respectively. For the step of crosslinking, two amounts of MTGase (30 and 60 U/g collagen) were added to the collagen solution and then incubated at different temperatures (20, 25, and 30 °C) for 12 h, respectively. After stopping the reaction by freezing at −40 °C, mixtures were lyophilized to obtain porous collagen matrices.

2.2. Viscometry of enzymatic crosslinked collagen gel solution

The rheological strength of the collagen solution was measured using a viscometer (DV-II, Brookfield, USA). Collagen solutions were prepared at various pH values. Soon after the original viscosity of collagen solution was determined, MTGase was added and incubated at a designed temperature for 6 h. At the end of the incubation, the final viscosity was measured. The increase ratio (V%) was calculated as

$$V(\%) = [(V_f - V_0)/V_0] \times 100,$$

where V_0 and V_f are the viscosity measured before and after enzymatic treatment, respectively.

2.3. Characterization of enzymatic crosslinked collagen matrices

A dynamic mechanical analyzer (DMA7e, Perkin-Elmer, USA) was used to measure the mechanical strength of the collagen matrix by recording the time–modulus curve. The tested sample was mounted on a stainless steel, parallel extension kit, and measurements were taken at ambient temperature. The initial applied force was 50 mN, with an extension rate of 200 mN/min. The modulus and stress of the collagen matrices at the break point were monitored. The tensile strength was calculated as the maximum stress reached before breakage of the material, as indicated by a sudden decrease in the recorded load.

The denaturation temperature (T_d) of MTGase-treated collagen matrices was measured by a differential scanning calorimeter (Perkin-Elmer DSC pyres-1, USA). Samples at 5 ± 2 mg were sealed in aluminum pans, and empty pans were used as references. Measurements were performed from 30 to 250 °C with a heating rate of 20 °C/min, and an average of triplicate was reported. Meanwhile, collagen matrix samples were coated with gold and investigated with a scanning electron microscope (Hitachi-S2400, Japan). The content of free amine groups was determined by the modified method of Bubnis [23]. The absorbance was measured at 345 nm. The crosslinking degree was calculated by the following equation:

$$\text{crosslinking degree} = 1 - (\text{absorption}_s/\text{mass}_s) \times (\text{absorption}_{ncl}/\text{mass}_{ncl}),$$

where s is the sample and ncl is non-crosslinked.

2.4. In vitro cytotoxicity test

Human foreskins for the isolation of fibroblast were obtained from Taipei Medical University Hospital. Fibroblasts used in all experiments were isolated by a method previously described. The cytotoxicity of collagenous matrices was evaluated by the MTT assay and cell morphology in contact with the tested sample. First, collagen samples were cut into suitable sizes and sterilized by submersion in 75% alcohol. After being washed with PBS buffer to remove any residual alcohol, samples were soaked in DMEM until they reached equilibrium before use. Fibroblasts at a concentration of

4×10^4 cells/well were directly seeded into 12-well culture plates into which a collagen sample had been placed and then cultured for 48 h. Cell morphology was observed by optical microscopy. The reduction in cell viability under conditions of co-culture with the tested samples was measured using the MTT assay. Fibroblasts were seeded at a concentration of 4×10^4 cells/well into 12-well culture plates. Two hours after seeding, sterilized collagen matrix was placed into each well and cultured with the cells for 48 h. Those wells into which no tested matrix was placed were used as the control. At the end of culture, the yellow tetrazolium MTT solution was added and incubated for 3 h until a purple precipitate was visible. The absorbance of each well was recorded at 550 nm.

3. Results

3.1. Viscosity

The viscosity of collagen solutions treated with various conditions was measured before and after treatment, respectively, and then the increase ratio was calculated. As presented in Fig. 1, the addition of MTGase to collagen solutions resulted in a net increase in viscosity. Furthermore, the increase in the ratio of viscosity was promoted by increasing the amount of MTGase. With a rise in the pH value, the increase ratios of viscosity were augmented in the matrices incubated at 20 and 25 °C. Of all the pH values, the viscosity of the collagen solution at pH 6 demonstrated the highest increase. It is noteworthy that, on the contrary, regardless of pH values or enzyme amounts, the viscosity of the collagen solutions incubated at 30 °C displayed a marked drop.

3.2. Crosslinking degree

In agreement with the results for viscosity (Fig. 2), the crosslinking degree increased with an increasing amount of enzyme. As the pH value of the collagen solution increased, a higher resultant crosslinking degree was obtained. However, with regard to the effect of incubation temperature, the crosslinking degree of the matrices incubated at 25 °C was higher than those for the other two corresponding sets of matrices. The maximum crosslinking degree (70%) was observed for matrices treated with MTGase at a ratio of 60 U/g collagen in pH 6 collagen solutions and incubated at 25 °C. On the other hand, even at pH 3, although the crosslinking degree was only around 29–46%, it still implies that MTGase retained some crosslinking activity.

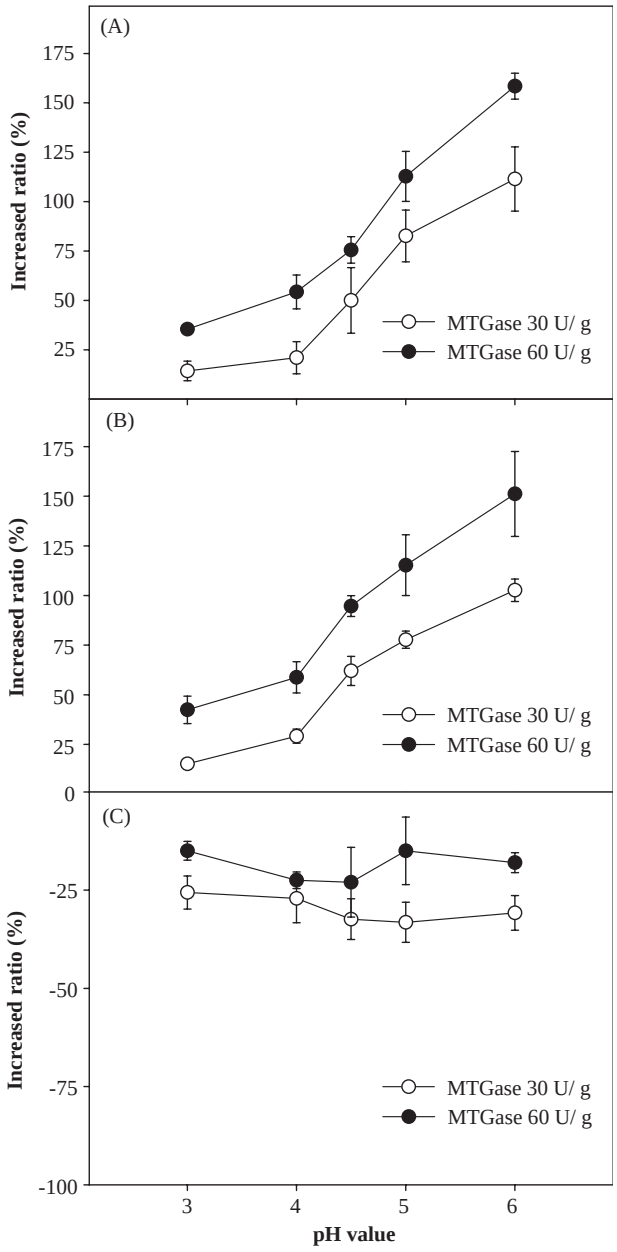

Fig. 1. Increased ratio of viscosity of MTGase-treated collagen solutions. Collagens were dissolved in acetic acid solutions of various pH values (pH 3, 4, 4.5, 5, and 6) to prepare collagen solutions and then incubated at (A) 20, (B) 25, and (C) 30 °C with the adding of two amounts of MTGase (30 and 60 U/g collagen), respectively ($n = 5$, mean ± SD). The incubation times were all set at 12 h.

3.3. Characterization of enzymatic crosslinked collagen matrices

As shown in Fig. 3, the resultant matrices crosslinked at low pH values of pH 3 and 4 exhibited higher tensile strengths than those at high pH values. However, this observation is contrary to the result for crosslinking degree, that is, a high crosslinking degree was observed

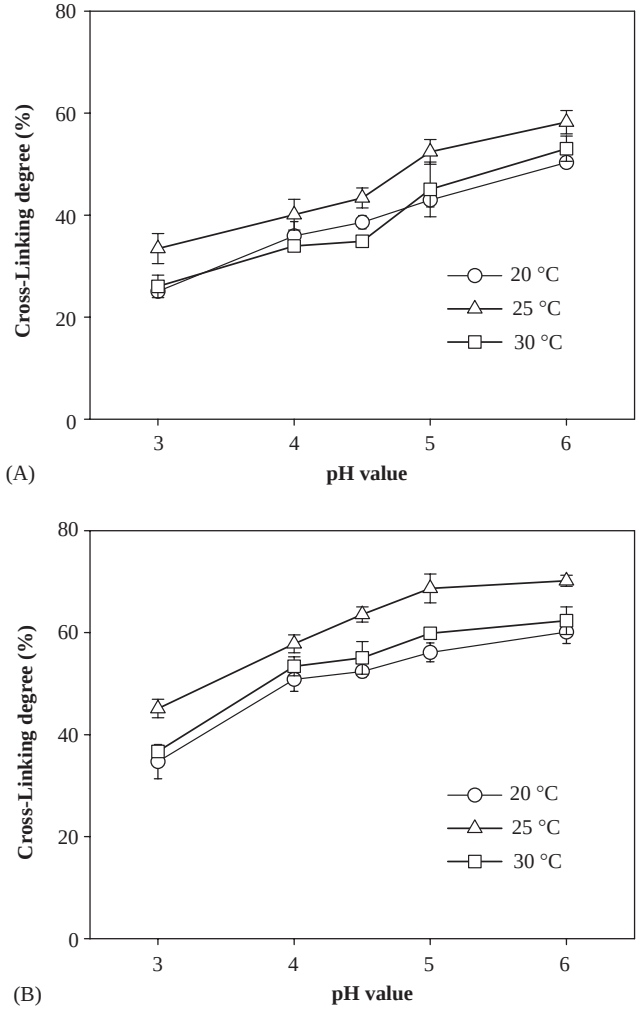

Fig. 2. Crosslinking degree of MTGase-treated collagen matrices. Matrices were treated with (A) 30 and (B) 60 U/g MTGase ($n = 5$, mean ± SD). The incubation times were all set at 12 h.

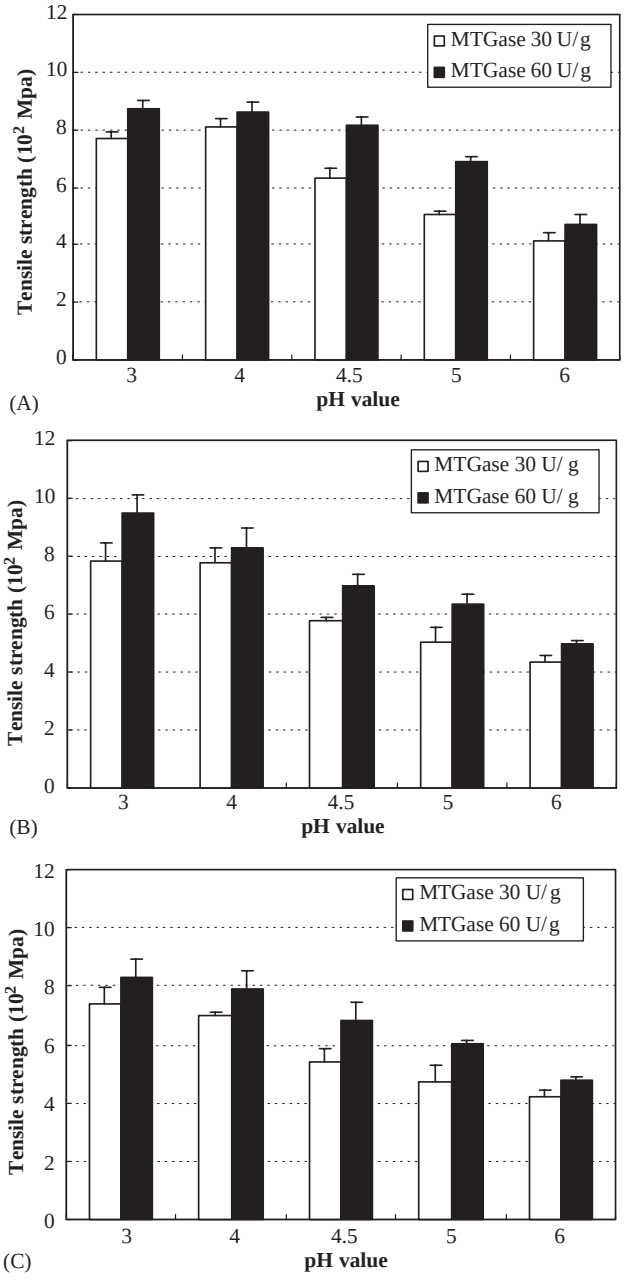

Fig. 3. Tensile strength of MTGase-treated collagen matrices. Matrices were incubated at (A) 20, (B) 25, and (C) 30 °C ($n = 5$, mean ± SD). The incubation times were all set at 12 h. A significant difference ($p < 0.05$) was noted between MTGase amounts.

for the resultant matrices crosslinked at high pH values. Except for the matrices incubated at 30 °C, the tensile strength of the matrices incubated at 20 and 25 °C increased as the amount of MTGase added increased.

Denaturation temperature (T_d) values of matrices incubated at 25 °C are summarized in Table 1, and typical DSC profiles in this study are shown in Fig. 4. It is obvious that the T_d values of matrices treated with enzyme all shifted toward higher temperatures. Statistical analysis indicated significant differences in T_d values between all enzyme-treated collagen matrices and untreated matrices. In addition, T_d values of matrices produced from collagen solutions having a pH value of 5 and 6 were significantly higher than those of other matrices. The T_d of the untreated collagen matrices recorded was around 113 °C, and the highest T_d was around 186 °C.

The cross-sectional morphology of matrices incubated at 25 °C was examined by SEM (data not shown). According to the SEM images, untreated collagen matrices displayed orderly and bigger pore sizes as compared to enzyme-treated matrices. There was no obvious difference among treated collagen matrices with respect to pore size and structure, except for matrices produced from the collagen solution with a pH value of 6, which showed irregular morphology and enlarged pore size.

Table 1
Denaturation temperatures of the MTGase-treated matrices incubated at 25 °C for 12 h

pH	MTGase (U/g)	
	30	60
3.0	172±3.6	175±2.6
4.0	174±2.6	176±2.5
4.5	171±2.5	172±4.8
5.0	183±5.1*	184±3.4*
6.0	180±4.6*	186±5.7*

A statistical difference, $p<0.05$, is marked with asterisk.Td values represented are mean±SD ($n=3$).

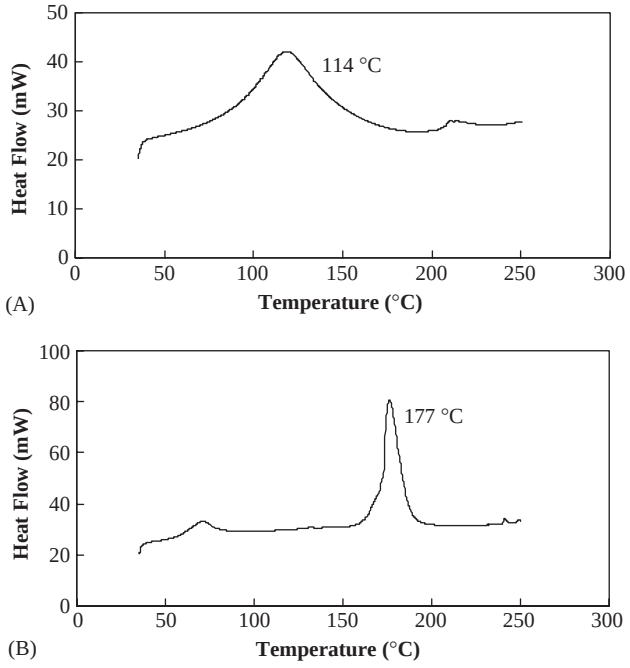

Fig. 4. DSC profiles of collagen matrices: (A) untreated collagen matrix and (B) collagen matrix treated with 60 U/g MTGase in a pH 4 collagen solution and incubated at 25 °C.

3.4. Evaluation of cytotoxicity

To evaluate the cytotoxicity, matrices incubated at 25 °C were tested. According to observations under light microscopy, human dermal fibroblasts in direct contact with enzyme-treated collagen matrices showed typical shuttle-like morphology compatible with their surroundings, as presented in Fig. 5. No abnormal morphology or cellular lysis was detected. Concerning the MTT assay (data not shown), the absorbance of the resultant formazan crystals among variously treated collagen matrices showed no significant differences. It revealed that collagen matrices crosslinked with various MTGase concentrations, even at a concentration of 60 U/g, showed no signs of reduced cell viability.

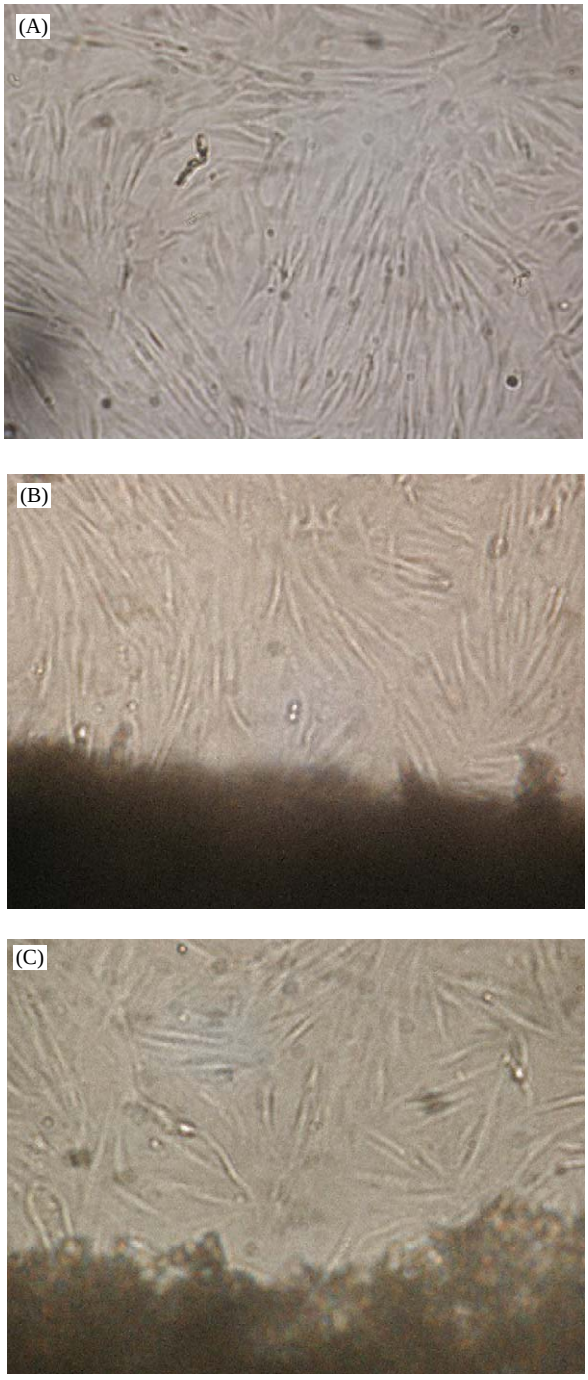

Fig. 5. Light micrographs of fibroblasts cultured for 48 h: (A) control; and fibroblasts in contact with the matrices crosslinked using 30 U/g MTGase (B) and 60 U/g (C). The dark area showed underneath (B) and (C) was matrices.

4. Discussion

According to previously published reports, MTGase is regarded as being stable over a wide range of pH values. While the optimum pH is around 5–8, even at pH 4 or 9, MTGase still displays some catalytic activity. The optimum reaction temperature for MTGase is

about 37 °C, but it is able to express appreciable activity even at a temperature of as high as 70 °C or as low as 10 °C. Thus, in order to investigate the processing factors that influence enzyme activity, we set the pH range for collagen solutions from pH 3 to 6 and the temperature range for the reaction from 20 to 30 °C. Crosslinked collagen solutions have been shown to be more resistant than non-crosslinked solutions; that is, the viscosity of collagen solution increases with the level of crosslinking. Obviously, as the results revealed, MTGase did exhibit a crosslinking action that raised the viscosity of the collagen solutions, and as the amount of enzyme increased, the solution became more viscous. Since the solubility of collagen differs under various values of pH, thus affecting viscosities, we used the increase ratio of each solution to depict the extent of crosslinking action. When the pH approached neutral, the increase ratio of viscosity was augmented. This was because the enzyme showed optimum activity around the pH range of 5–8. Meanwhile, it is noteworthy that all of the final viscosities of treatments incubated at 30 °C were markedly decreased. Some aggregates appeared and water escaped from the collagen fiber bundles, which caused a drop in viscosity. Likewise, a previous report demonstrated that up to 30 °C, the yield value of collagen dispersions decreased due to precipitation of the collagen fiber [24]. The phenomenon of a resultant drop in viscosity indicates that the temperature effect is more prominent than the enzymatic crosslinking effect under these conditions.

It is known that crosslinking reinforces the tensile strength of collagen matrices. It is evident that, as a whole, the tensile strength of the matrices increased after treatment with MTGase. Among the treatments used in this study, high tensile strength was observed for those matrices crosslinked at a pH value of 3 and 4, whereas the crosslinking degree of these matrices was lower than those of other tested matrices. The reason can be clarified by the solubility difference of collagen under various pH conditions. At pH 3, although the enzyme activity was appreciable, the collagen fibers were almost completely bound with water, and hence formed a homogeneous solution. On the contrary, collagen fibers did not fully dissolve in solutions at pH 5 or 6, creating a heterogeneous solution, which also resulted in unequal development of enzymatic cross-linkages. This was also confirmed by the SEM images. The cross section of those matrices crosslinked at pH 3 displayed a dense small pore size and a regular structure, whereas the pore sizes of matrices crosslinked at pH 6 were enlarged, and the structure was irregular which caused the material to break easily, thus reducing its tensile strength.

T_d is affected by numerous factors such as genetic type, age, and the number and arrangement of cross-linkages [25]. To examine the crosslinking effect of MTGase, thermal denaturation of collagen matrices was characterized by differential scanning calorimetry. The T_d at which the unfolding of the protein structure takes place here was determined as the peak value of the corresponding endothermic phenomenon in the DSC thermogram. A broad, endothermic peak located at around 113 °C was observed for untreated collagen matrices. It was documented that this peak is attributed to the complex thermo-transition that comprises disruption of protein/water interactions, rupture of hydrogen bonds, and the evaporation and vaporization of the bound water [26]. Furthermore, in comparison with the untreated matrices, the T_d values of the corresponding matrices shifted toward higher temperatures and their endothermic peaks showed a narrow and sharp pattern which was also reflected in a decrease in enthalpy (data not shown). This can be ascribed to the increase in cross-linkages that break exothermically and the situation in which as the number of cross-linkages increases, less water can be bound [27]. On the other hand, results showed that T_d values of matrices produced from pH 5 and 6 collagen solutions were higher than those of the other matrices tested. This can probably be explained by the pH of the collagen solution being at an optimal condition for enzyme activity. The results for cross-linking degree which had a similar trend also confirm this explanation. As for the influence of the amounts of MTGase, a recent study reported that with an increase in the amount of enzyme, the T_d values increased [28]. However, we observed no difference regarding the effect of the amount of enzyme among treatments.

As reported by many, the potential toxicity of modified collagen matrices can be attributed to the crosslinking reagents and their by-products [29,30]. Although MTGase derived from a variant of *Streptoverticillium mobaraense* has been commercially available for years, its potential cytotoxicity is still a major concern. In our study, the in vitro cytotoxicity of enzymatically crosslinked collagen matrices was evaluated on the basis of cell morphology and cell viability. The results indicated that human fibroblasts grown with the tested collagen matrices did not stay in a round shape and did not stop proliferating. The cells grew well within the materials and showed a normal morphology. In microscopic observations, it seemed that neither MTGase, the crosslinking agent, itself nor its by-product affected the cells. The MTT assay was used herein to measure cell viability. Only cells that are metabolically normal can turn the tetrazolium salts into purple crystals. Compared with the controls, all of the tested matrices, even matrices treated with a high concentration (60 U/g collagen) of MTGase, showed no significant differences in absorbance, that is, the matrices being in direct contact with fibroblasts did not lead to apoptosis or necrosis. Thus, as proven by the above evaluations, the cytotoxicity of the matrices was acceptable.

5. Conclusions

In conclusion, improvements in porcine collagen matrices can be achieved by crosslinking using microbial TGases. Like other chemical or physical methods commercially available, this enzymatic crosslinking method was found to be easy to use during the in situ crosslinking process and was proven to be non-cytotoxic in the examination of cytotoxicity.

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